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Carbapenem Susceptibility Testing Errors Using Three Automated Systems, Disk Diffusion, Etest, and Broth Microdilution and Carbapenem Resistance Genes in Isolates of *Acinetobacter baumannii-calcoaceticus* Complex▽

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The *Acinetobacter baumannii-calcoaceticus* complex (ABC) is associated with increasing carbapenem resistance, necessitating accurate resistance testing to maximize therapeutic options. We determined the accuracy of carbapenem antimicrobial susceptibility tests for ABC isolates and surveyed them for genetic determinants of carbapenem resistance. A total of 107 single-patient ABC isolates from blood and wound infections from 2006 to 2008 were evaluated. MICs of imipenem, meropenem, and doripenem determined by broth microdilution (BMD) were compared to results obtained by disk diffusion, Etest, and automated methods (the MicroScan, Phoenix, and Vitek 2 systems). Discordant results were categorized as very major errors (VME), major errors (ME), and minor errors (mE). DNA sequences encoding OXA beta-lactamase enzymes ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-58-like}}$, and $bla_{\text{OXA-51-like}}$) and metallo-β-lactamases (MBLs) (IMP, VIM, and SIM1) were identified by PCR, as was the *KPC2* carbapenemase gene. Imipenem was more active than meropenem and doripenem. The percentage of susceptibility was 37.4% for imipenem, 35.5% for meropenem, and 3.7% for doripenem. Manual methods were more accurate than automated methods. $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like}}$ were the primary resistance genes found. $bla_{\text{OXA-58-like}}$, MBLs, and *KPC2* were not present. Both automated testing and manual testing for susceptibility to doripenem were very inaccurate, with VME rates ranging between 2.8 and 30.8%. International variability in carbapenem breakpoints and the absence of CLSI breakpoints for doripenem present a challenge in susceptibility testing.

The *Acinetobacter baumannii-calcoaceticus* complex (ABC) is a common nosocomial pathogen with increasing carbapenem resistance (19). A survey of 55,330 ABC isolates obtained from The Surveillance Network (TSN) revealed a 3.7-fold increase in carbapenem resistance, from 20.6% in 2002 to 49.2% in 2008 (17). At Brooke Army Medical Center, where our study was conducted, a rapid increase in resistance to four first-line antimicrobial classes, including carbapenems, was seen from 2004 to 2006 (14).

As antimicrobial resistance increases, accurate susceptibility testing to guide therapeutic options is essential. Although most clinical microbiology laboratories rely heavily on automated systems as the primary method of susceptibility testing, several studies have reported significant error rates in determining the susceptibilities of ABC isolates to various antimicrobials using automated testing platforms (1, 12, 15). The accuracy of methods for testing susceptibility to carbapenems has not been extensively evaluated for multidrug-resistant (MDR) ABC isolates.

In this study, we evaluated the accuracies of automated and manual methods in determining the susceptibilities of clinical ABC isolates to three carbapenems by comparison to broth microdilution (BMD) as the reference method, and we evaluated these isolates for the presence of various resistance genes.

MATERIALS AND METHODS

Bacterial isolates. A total of 107 nonrecurring single-patient ABC isolates from blood and wound infections collected at Brooke Army Medical Center between 2006 and 2008 were studied. Frozen cultures were passed twice on blood agar plates (Remel, Lenexa, KS) before testing. Quality control strains used for antimicrobial susceptibility testing included *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA).

Bacterial identification. Isolates were identified using the Vitek 2 (bioMérieux, Durham, NC) and Phoenix (Becton, Dickinson and Co., Franklin Lakes, NJ) automated microbiology systems. All isolates were identified as ABC by clinical and molecular methods as previously described (1, 22).

PFGE. Clonal relationships were assessed by pulsed-field gel electrophoresis (PFGE) following the PulseNet protocol of the Centers for Disease Control and Prevention, with modifications for *Acinetobacter*. The pulsed-field types of these isolates have been described previously (1).

Antimicrobial susceptibility testing. The susceptibilities of the isolates to imipenem (Merck, Whitehouse Station, NJ), meropenem (AstraZeneca Pharmaceuticals, Wilmington, DE), and doripenem (Shionogi & Co., Osaka, Japan), and the MICs of these antimicrobials, were determined by disk diffusion and broth microdilution, the reference method (range, 0.015 μg/ml to 16 μg/ml), according to the methods and interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI) and by Etest (AB bioMérieux, Solna, Sweden) according to the manufacturer's instructions (3, 18, 23).

Antimicrobial solutions for broth microdilution testing were prepared from powders provided by the manufacturers. The bacterial densities of the inoculum solutions were verified by colony counts. Automated susceptibility testing was

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TABLE 1. MICs and error rates obtained for 107 single-patient clinical isolates of the *Acinetobacter baumannii-calcoaceticus* complex tested for susceptibility to imipenem, meropenem, and doripenem by manual and automated methods compared to BMD by using CLSI, FDA, and EUCAST criteria

Drug and method[a]	MIC (μg/ml)		Result[b] using the following criteria:											
			CLSI[c]				FDA[d]				EUCAST[e]			
	50%	90%	% S	% VME	% ME	% mE	% S	% VME	% ME	% mE	% S	% VME	% ME	% mE
Imipenem														
DD			39.6	0.0	0.9	1.9	39.6	0.0	0.9	1.9	31.8	17.8	2.8	NA
ET	≥32	≥32	39.3	0.0	0.9	3.7	39.3	0.0	0.9	3.7	13.1	5.6	6.5	NA
MicroScan	≥8	≥8	43.0	3.7	1.9	2.8	43.0	3.7	1.9	2.8	38.3	25.2	1.9	NA
Phoenix	≥8	≥8	41.1	1.9	0.9	2.8	41.1	1.9	0.9	2.8	37.4	25.2	0.9	NA
Vitek 2	≥16	≥16	39.3	0.9	0.0	4.7	39.3	0.9	0.0	4.7	38.3	25.2	0.9	NA
BMD	≥16	≥16	37.4				37.4				15.0			
Meropenem														
DD			38.3	0.0	0.0	3.7	38.3	0.0	0.0	3.7	8.4	0.9	12.1	NA
ET	≥32	≥32	36.4	0.0	0.0	3.7	36.4	0.0	0.0	3.7	19.6	4.7	4.7	NA
MicroScan	≥8	≥8	38.3	0.0	0.9	3.7	38.3	0.0	0.9	3.7	37.4	18.7	0.9	NA
Phoenix	≥8	≥8	39.2	1.9	0.9	4.7	39.2	1.9	0.9	4.7	35.5	17.8	0.9	NA
Vitek 2	≥16	≥16	39.3	0.0	0.9	4.7	39.3	0.0	0.9	4.7	36.4	17.8	0.9	NA
BMD	≥16	≥16	35.5				35.5				19.6			
Doripenem														
DD			NA	NA	NA	NA	35.5	30.8	0.9	NA	7.5	3.7	0.0	NA
ET	≥32	≥32	NA	NA	NA	NA	6.5	2.8	0.0	NA	6.5	2.8	0.0	NA
Vitek 2	≥8	≥8	NA	NA	NA	NA	21.5	17.8	0.0	NA	21.5	17.8	0.0	NA
BMD	≥16	≥16	NA				3.7				3.7			

[a] DD, disk diffusion; ET, Etest; Vitek 2, Vitek 2 cards (AST-GN24 card and AST-XN04 extension card).
[b] S, susceptible; VME, very major errors; ME, major errors; mE, minor errors; NA, not applicable (intermediate breakpoints for doripenem were not defined).
[c] CLSI breakpoints for imipenem/meropenem were as follows: for susceptibility, ≤4 μg/ml (≥16 mm); for intermediacy, 8 μg/ml (14 to 15 mm); for resistance, ≥16 μg/ml (≤13 mm) (3).
[d] FDA breakpoints were as follows: for imipenem/meropenem, ≤4 μg/ml (≥16 mm) for susceptibility and ≥16 μg/ml (≤13 mm) for resistance; for susceptibility to doripenem, ≤1 μg/ml (≥17 mm).
[e] EUCAST breakpoints were as follows: for imipenem, ≤2 μg/ml (≥23 mm) for susceptibility and ≥8 μg/ml (≤17 mm) for resistance; for meropenem, ≤2 μg/ml (≥21 mm) for susceptibility and ≥8 μg/ml (≤15 mm) for resistance; for doripenem, ≤1 μg/ml (≥21 mm) for susceptibility and ≥4 μg/ml (≤15 mm) for resistance (6).

performed with the Vitek 2 system for imipenem, meropenem, and doripenem by following the manufacturer's instructions using the AST-GN24 card and the AST-XN04 extension card (bioMérieux, Durham, NC). In addition, the MicroScan WalkAway (Siemens, Deerfield, IL) and BD Phoenix automated microbiology systems were used for imipenem and meropenem susceptibility testing by following the manufacturers' instructions and using MicroScan-Neg MIC type 32 panels and Phoenix ID-123 panels. The minimum concentrations of antibiotic required to inhibit the growth of 50% and 90% of the isolates tested (the MIC_{50} and MIC_{90}, respectively) were calculated for each agent. Discordances between the reference method (broth microdilution) and the other methods were tabulated as very major errors (VME) (reported susceptible when resistant), major errors (ME) (reported resistant when susceptible), or minor errors (mE) (reported intermediate when resistant or susceptible, or vice versa) (11). CLSI breakpoints were used to determine susceptibilities to imipenem and meropenem (3). The breakpoints defined by the U.S. Food and Drug Administration (FDA) and the European Committee on Antimicrobial Susceptibility (EUCAST) were used for doripenem, since CLSI breakpoints for *Acinetobacter* have not been defined (6, 18). Error rates were compared to allowable tolerances specified by the FDA for susceptibility testing devices: a very major error rate of <1.5%, a major error rate of <3.0%, and an overall essential agreement of >90% of MICs within 1 doubling dilution of the MIC measured by a CLSI reference method (11). Minor error rates were not calculated for doripenem, since intermediate breakpoints were not defined.

Gene amplification and detection. Multiplex PCR assays were conducted to identify DNA sequences encoding OXA beta-lactamase enzymes ($bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, and $bla_{\text{OXA-58-like}}$) and metallo-beta-lactamases (IMP, VIM, and SIM1), as well as the *KPC2* carbapenemase gene. Genomic DNA extracted from each bacterial isolate was used as a template. Primers corresponding to previously published sequences for these genes were obtained commercially (Midland Certified Reagent Company, Midland, TX) (4, 20, 24). Each reaction mixture contained 10 μl of 5 PRIME MasterMix (2.5×), 1 μl each of 12.5-pmol/μl forward and reverse primers, and 20 ng of template DNA in a final volume of 25 μl. An initial denaturation step was carried out at 94°C for 5 min, followed by 30 amplification cycles for OXA genes and 36 cycles for IMP, VIM, and SIM genes. Each cycle consisted of denaturation for 30 s at 94°C, annealing for 40 s at 52°C, and extension for 50 s at 72°C, followed by a final extension for 5 min at 72°C. For amplification of the *KPC2* gene, 0.5 μl of 2.5 mM Mg^{2+} was added to the solution with a final volume of 25 μl, and initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of amplification. Each cycle consisted of denaturation for 30 s at 95°C, annealing for 1 min at 52°C, and extension for 1 min at 72°C, followed by a final extension for 5 min at 72°C. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Amplified DNA fragments were identified by estimation of their base pair lengths using a DNA ladder and were compared to positive controls if available. Positive controls included $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-51-like}}$, and bla_{KPC2}. No positive controls were available for the bla_{IMP}, bla_{VIM}, and bla_{SIM1} genes, and thus, reaction products were identified by their estimated base pair lengths. The relationship between carbapenem susceptibility and the presence of resistant genes was examined.

RESULTS

Characterization of clinical isolates. All isolates were identified as members of the ABC by using the Vitek 2 and Phoenix instruments. The $bla_{\text{OXA-51-like}}$ gene, characteristic of ABC, was present in all isolates.

Carbapenem susceptibility and error rates. Imipenem was the most active carbapenem by use of the CLSI and FDA breakpoints. By application of the EUCAST breakpoints, meropenem was more active than imipenem. Susceptibility to doripenem was 3.7% by use of the FDA and EUCAST breakpoints. The MIC_{90} was ≥16 μg/ml for all three agents (Table 1).

TABLE 2. Broth microdilution susceptibilities and MIC distributions for all 107 single-patient *A. baumannii-calcoaceticus* complex isolates and for isolates by the presence or absence of the $bla_{\text{OXA-23-like}}$ gene

Agent	Result for isolates[a]:								
	Total			With the $bla_{\text{OXA-23-like}}$ gene			Without the $bla_{\text{OXA-23-like}}$ gene		
	MIC (μg/ml)		% S	MIC (μg/ml)		% S	MIC (μg/ml)		% S
	50%	90%		50%	90%		50%	90%	
Imipenem	≥16	≥16	37.4	≥16	≥16	0	4	≥16	78.4
Meropenem	≥16	≥16	35.5	≥16	≥16	0	4	≥16	72.5
Doripenem[b]	≥16	≥16	3.7	≥16	≥16	0	4	≥16	3.7

[a] Among the total number of isolates, 56 (52.3%) had the $bla_{\text{OXA-23-like}}$ gene and 51 (47.7%) lacked it. S, susceptible.
[b] FDA breakpoints were applied (≤1 μg/ml).

By use of the CLSI and FDA breakpoints for imipenem and meropenem, the VME rate observed for automated methods ranged from 0.0 to 3.7% (Table 1). The ME rate of disk diffusion and the Etest for imipenem was 0.9%; no ME were found with manual testing for meropenem susceptibility (Table 1). Automated and manual testing VME rates ranged from 0.9 to 25.2% for imipenem and meropenem by use of the EUCAST breakpoints (Table 1). The Vitek 2 VME rate was 17.8% for doripenem by application of the FDA and EUCAST breakpoints. The VME rate for manual testing ranged from 2.8% to 30.8% for doripenem.

Beta-lactamase genes. $bla_{\text{OXA-51-like}}$ genes were identified in all isolates, consistent with their biochemical identification as ABC. Fifty-six isolates contained $bla_{\text{OXA-23-like}}$ genes; all of them were carbapenem resistant (Table 2). $bla_{\text{OXA-23-like}}$ genes were associated with elevated carbapenem MICs and lower susceptibility to carbapenem than that of isolates without the gene. $bla_{\text{OXA-24-like}}$ genes were present in only four isolates, all of which were resistant to all three carbapenems. $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like}}$ genes were found in 60 of 64 imipenem-resistant isolates. MBL-encoding genes, *KPC2*, and $bla_{\text{OXA-58-like}}$ genes were not detected in any of the isolates. For 4 of the imipenem-resistant isolates, no resistance gene (other than $bla_{\text{OXA-51-like}}$) was identified.

DISCUSSION

ABC is an important nosocomial pathogen seen with increasing frequency throughout medical facilities. The ability of ABC to acquire extensive drug resistance has become a major concern to the medical community due to limited therapeutic options and challenges for infection control (13). In 107 clinical ABC isolates, we observed carbapenem susceptibilities ranging between 3.7% and 37.4% by BMD; imipenem was slightly more active than meropenem. Doripenem was significantly less active when FDA breakpoints were applied (3.7%) and only slightly less active (33.7%) when the CLSI breakpoints defined for imipenem and meropenem were used. We found automated systems to be less accurate than manual methods for determining susceptibilities to imipenem and meropenem, and we observed unacceptable ME rates for the MicroScan and Phoenix systems. Both automated and manual methods were inaccurate compared to BMD for doripenem by use of FDA breakpoints. Carbapenem-hydrolyzing oxacillinase genes, which are associated with carbapenem resistance, were present in 60 isolates.

We found imipenem to be slightly more active than meropenem when tested by the reference method, broth microdilution. The increased *in vitro* activity of imipenem compared to those of meropenem and doripenem has been reported previously for ABC. In a large study of nonfermentative bacilli, including 3,844 ABC isolates, 69.4% of ABC isolates were susceptible to imipenem, 66.6% to meropenem, and 49.9% to doripenem, supporting the increased activity of imipenem and the decreased activity of doripenem (2). In contrast, a prior study found doripenem to be more active than imipenem and meropenem against 24 carbapenem-resistant ABC isolates (20.8% of isolates were susceptible to doripenem, 16.7% to imipenem, and 4.2% to meropenem) by BMD (10). It should be noted that these investigators used the CLSI breakpoints defined for imipenem and meropenem (susceptibility breakpoint, ≤4 μg/ml) to determine doripenem susceptibility (10). In a more recent study, doripenem was found to have lower activity against ABC than imipenem and meropenem, which is consistent with our results (5). In that study, the FDA-cleared breakpoints in the package insert for doripenem (susceptible at ≤1 μg/ml) and the CLSI-defined breakpoints for meropenem and imipenem (susceptible at ≤4 μg/ml) were used, consistent with our methods. If we applied the higher doripenem breakpoints used by Jones et al. to our isolates, the percentage of isolates susceptible to doripenem would have increased from 3.7% to 33.6%, which would have been comparable to those for imipenem and meropenem (10). This dramatic difference highlights the impact of inconsistent breakpoints on antibiotic susceptibility testing. If the same breakpoints were used for all three carbapenems, their activities would be equally poor in this highly carbapenem resistant ABC population.

Automated susceptibility testing methods are widely used in clinical settings because of their efficiency and convenience. However, the accuracy of automated methods for testing the susceptibilities of ABC isolates to carbapenems has been varied (8, 12, 16). Our study documents error rates in carbapenem susceptibility testing among automated systems that exceed the U.S. FDA standards required for device approval (11). The Vitek 2 method was the only automated susceptibility method in our study that satisfied FDA criteria for approval when used for imipenem and meropenem. These results are consistent with a prior study of 192 strains of nonfermenting gram-negative bacilli (including 25 *Acinetobacter* strains) that observed a VME rate of 0.7% for the Vitek 2 instrument (8). In contrast, a study examining 25 strains of ABC observed a VME rate of

4.0% for imipenem susceptibility testing with the Vitek 2 instrument (12). We observed a significant VME rate with the Vitek 2 instrument for doripenem susceptibility testing using the FDA-approved breakpoints.

The MicroScan system had the highest VME rate for imipenem susceptibility testing (2.8%). This was significantly lower than the 25% VME rate reported by a previous study (15). A VME rate of 1.9% was noted with the Phoenix automated system for both imipenem and meropenem, which is above the acceptable rate of ≤1.5%. In contrast, in a study that included 9 ABC isolates, no VME or ME were reported with the Phoenix system for imipenem and meropenem susceptibility testing (16). Our error rates for testing susceptibility to imipenem and meropenem by disk diffusion and the Etest were within an acceptable range. In contrast, the accuracy of manual testing for susceptibility to doripenem was significantly lower. When we applied the current CLSI carbapenem breakpoints to doripenem, the ME rate for both disk diffusion and the Etest was 1.9%. However, when we applied the FDA breakpoints for doripenem, the VME rates for the Etest and disk diffusion were 2.8% and 30.8%, respectively. The significant VME rate observed with disk diffusion by using the FDA breakpoints was reduced to 3.7% when we applied the EUCAST breakpoint of ≥21 mm. A further reduction in the error rate to 0.9% was noted when we used a breakpoint of ≥24 mm, which was derived from the correlation between the disk diffusion diameter and MICs for 810 ABC isolates (7). These observations once again highlight the importance of establishing standardized breakpoints. Based on our findings and previously published data, manual methods are superior for testing the susceptibility of ABC isolates to carbapenems.

Carbapenem resistance in ABC isolates has been attributed primarily to oxacillinases (19). We identified a mechanism of carbapenem resistance in 60 out of 64 imipenem-resistant isolates. We identified $bla_{\text{OXA-23-like}}$ genes in 56 of our isolates, all of which were carbapenem resistant. The association between oxacillinases and carbapenem resistance is consistent with the findings of prior studies (9, 21). Carbapenem resistance was associated with $bla_{\text{OXA-24-like}}$ and $bla_{\text{OXA-58-like}}$ genes in 11% and 12%, respectively, of 75 ABC isolates from the Walter Reed Army Medical Center (9). In contrast, we found $bla_{\text{OXA-24-like}}$ genes in only four of our isolates (3.7%), and $bla_{\text{OXA-58-like}}$ genes were not detected.

MBLs and *KPC2* were not identified in any of our isolates. Four of the imipenem-resistant isolates did not have the $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, or $bla_{\text{OXA-58-like}}$ gene. The proposed mechanisms of resistance in these four isolates include increased expression of $bla_{\text{OXA-51-like}}$ oxacillinase induced by the promoter sequence ISA*ba*1, modified porin channels, or penicillin binding proteins and efflux mechanisms (19).

This study has several limitations. The Vitek 2 system was the only automated susceptibility platform for which cards containing doripenem were available. The lack of an international consensus regarding carbapenem susceptibility breakpoints for ABC poses an additional difficulty. The imipenem and meropenem breakpoints for ABC established by EUCAST are 2 μg/ml and 8 μg/ml, compared to the current CLSI breakpoints of 4 μg/ml and 16 μg/ml. When we applied the EUCAST breakpoints to our isolates, imipenem susceptibility decreased from 37.4% to 15.0%. If we applied the CLSI breakpoints defined for imipenem and meropenem to doripenem, the percentage of isolates found susceptible would have been 33.6%, compared to only 3.7% by use of the breakpoints defined in the package insert.

In summary, more than 60% of the clinical ABC isolates tested were resistant to carbapenems and were $bla_{\text{OXA-23-like}}$ strains. Based on our ABC resistance profile, empirical carbapenem monotherapy for ABC at our institution would provide inappropriate coverage. To improve accuracy, we recommend manual susceptibility testing for ABC isolates and advise caution in interpreting the results of antimicrobial susceptibility testing methods for doripenem. Until the CLSI defines breakpoints for doripenem, medical centers within the United States should apply the FDA breakpoints for doripenem susceptibility testing. Furthermore, an international standard for carbapenem ABC breakpoints should be established to address the large differences in susceptibility results and in the accuracy of susceptibility testing methods.

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